SICLOPPS Derived Libraries as a Means to Rescue Complex Formation Inhibited by Cancer Mutations

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By

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Abstract

According to the American Cancer Society, one in four males and one in five females have a 100% lifetime risk of dying from cancer. Cancer is a major burden on modern society, from stealing away precious years of health to putting stress on an already over burdened health care system. While some drugs might work better on general types of cancer, it is plausible to create cures for cancers based on specific mutations found within key regulatory proteins. Two complexes that are relevant to cancer biology, and are prime targets for drug modification, include a p53/DNA complex and a BARD1/BRCA1 protein-protein dimer. Both complexes are commonly mutated in human cancers and many of those mutations inhibit formation of signaling complexes required for keeping cancerous growth in check. Using libraries of small, cyclic, genetically encoded molecules, made by the SICLOPPS method, it is possible to seek scaffolds for future cancer drugs. Before being able to screen with confidence, however, it is first necessary to study SICLOPPS molecule interactions on the functional screens used to monitor p53/DNA and BRCA1/BARD protein complex association and formation in order to learn how to optimize the screening process. In conclusion, insight was gained into how to use novel functional assays with SICLOPPS derived libraries and in the future large numbers of SICLOPPS molecules will be screened against important cancer associated complexes in order to rescue complex formation.

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Chapter 1 - Introduction

Mutations that inhibit formation of important cancer-check point protein complexes

Protein interactions govern much of the biology that supports life. Proteins perform many basic functions including acting as signal transducers that moderate and support the metabolism of functioning organisms. However, when these protein interactions fail, a link is broken that can often lead to organismal catastrophe. The interactions of BRCA1, and its protein partner, BARD1, along with p53 and its ligand DNA, hold important functions within our cells that are absolutely necessary. When these complexes can no longer form, usually through mutation that causes perturbations within the proteins, dire consequences are the result.

The advances in protein chemistry yields hope for a solution to these problems. We propose a method of finding molecules that act as protein chaperones, helping to restore order to cellular function when mutations occur. Through a peptide library, made by the SICLOPPS method, we hope to discover drug scaffolds that could help in the fight against cancer.

A. P53

i. The p53 protein significance in cancer development

The study of the p53 protein has had a large impact on cancer biology since its discovery in 1979. The protein itself was discovered independently by two different groups when it was found that p53 complexes with the SV40 large T-antigen.[1,2] Since it was discovered and implicated as a component in human cancer development thirty years ago, the gravity of the role of p53 has emerged in great detail. The knowledge base has evolved greatly over those thirty years, as it was first thought p53 acted as an oncogene in tumor formation, but since the mid 1990's it has been clarified that wild-type p53 acts as a tumor suppressor *in vivo*.[3] In fact, p53 is inactivated in at least fifty percent of all human cancers.[4] Besides being found in human cancers, tumors that contain p53 missense mutations are associated with poor prognosis and long-term survivability. This is largely due to resistance to chemotherapy that p53-mutation positive cells commonly display. It is hypothesized that chemotherapeutic drugs induce apoptosis in cancerous cells by channeling p53 apoptotic pathways, which cannot be activated in cells with existing p53 mutations that result in functional loss.[5] Mutations that inactivate p53 occur late in tumorogenesis, usually occurring after many other genomic instabilities and other allelic losses arise.[6] It has been indicated that p53 inactivation is a rate limiting step for cancer growth in part to the late loss of function observed in tumorogenesis. Multiple groups have implicated that p53 reactivation in cancer cells leads to cancer suppression *in vivo*.[7,8] A well-known familial cancer syndrome called Li-Fraumeni Syndrome involved passing on germ line mutations of p53 between family members. Families that carry Li-Fraumeni Syndrome show remarkably increased occurrences of diverse types of tumors at early ages.[9] Similarly, mice homozygous for the p53 null allele have been shown to develop cancer by six months of age while very small numbers of

homozygous and wild-type mice develop any type of cancer in the same time period.[10] The Li-Fraumeni Syndrome germlines and the p53 deficient mice points to the overall importance of p53 as a tumor suppressor *in vivo*. Small scale gene therapy trials have exploited this and has shown promising results using retrovirus-mediated p53 gene transfer into lung cancer tumors during clinical trials.[11] However, rescuing p53 function via gene therapy is likely years away from being implemented as a panacea for cancer due to complications in the immune system response to vectors of wild-type p53 gene transfer and other limiting factors.[12] This does show that restoring the function of p53 can lead to tumor suppression.

ii. The role of p53 in vivo

Activation of p53-dependent pathways depend on different kinds of cellular stress occurring in a cell. The first type of cellular stress shown to activate p53 was DNA damage. Kuerbitz *et al.* gamma-irradiated to cause DNA damage in cell lines that didn't contain endogenous p53 while also irradiating the same cell lines that had been transfected with wild type p53.[13] This resulted in cell growth being stopped in the G1 phase in the cells containing the wild-type allele while the null allele cells did not stop replication. This indicated the important role of p53 to DNA damage. Additional cellular stresses have been shown to activate p53 cell senescence pathways, including oncogene activation, ribosomal stress, loss of cell to cell contacts, and hypoxia.[14] Many different pathways of activation through post-translational modification have been suggested for p53. These include phosphorylation by stress-response kinases such as ATM, ATR, Chk1, and CHk2; Acetlyation by the p300/CBP complex, and stabilization through interaction of various other proteins including BRCA1, E2F1, and pRB.[15]

Unstressed, normal cellular environments have very low concentrations of p53, due to a short half-life that is tightly controlled by the MDM2 protein. MDM2 acts directly on p53 as an

E3 ubiquitin ligase.[16] MDM2 actively appends ubiquitin to p53 which targets it for destruction by proteolysis in the cell. MDM2 also indirectly controls p53 transduction via a negative autoregulatory feedback loop.[17]

The main function of active p53 is as a cell cycle regulator. One of the first experiments to suggest p53's activity as a cell cycle regulator was performed using colorectal cell lines, which lacked the p53 gene or contain missense mutations in p53, which showed a five to ten-fold decrease in proliferation in comparison to the same cell line that had not been transfected.[18] This seminal experiment gave some of the first results indicating that p53 plays a major role in cell cycle regulation. p53 has also been noted to play important roles in cellular apoptosis.[19] The activation of p53 allows it to carry out its main function as a transducer of important genes that encode important cell cycle regulators. Some of the important genes that p53 regulates includes the pro-apoptotic Bax genes, GADD45 genes which play a part in Cyclin B inhibition, and many others, including genes not yet characterized.[20] To activate many of these pathways, p53 has been found to bind a consensus binding site characterized by Baker *et al*.[18] The binding site is composed of two distinct copies, where each copy is called a half-site, of the 10 base pair motif of 5'-RRRC(A/T)(T/A)GYYY-3' with 0 to 13 base pairs between the copies, where R is a purine and Y is a pyrimidine. The symmetry of the sequence has been shown to allow four monomers of p53, meaning that p53 binds as a tetramer to the consensus binding sequence. In fact, it has been shown by Weinberg *et al.* that p53 binding to the consensus binding site is highly cooperative and that a fully stable DNA/p53 complex requires the full length DNA binding site along with four monomers of p53.[21] It has also shown that common cancer mutations can cause a lack of tetrameric complex formation within the consensus DNA binding site.[22] To understand why

many of these cancer mutations prevent complex formation, an understanding of the molecular structure of p53 is required.

iii. The Structure of p53

Human p53 is a 393 amino acid protein that has been historically divided into five different domains, albeit some functional properties overlap between these domains. The N-terminus of p53 is a very structurally plastic region that has not been found to have a tertiary structure.[23] Within the N-terminus of p53 exists two of the five functional domains found within p53, with the first 64 amino acids being the transactivation region and the following 65 to 92 amino acids the proline rich domain. Both regions play a role in the transcription of p53 regulated genes.[24] Residues 93-292 compose the DNA-binding region, the region that specifically targets the consensus binding site.[25] Amino acids 324 to 361 makeup the tetramerizaion domain of p53, which is important for formation of the homotetramer/DNA complex.[26] The fifth region, the regulatory domain, is composed of amino acids 362-392. It is a key domain that is often post-translationally modified for activation. Similarly to the N-terminus region, the regulatory domain is unstructured under physiological conditions and gains a secondary structure after binding to other proteins.[27] A concise review of the domains has been written by Viadiu.[28]

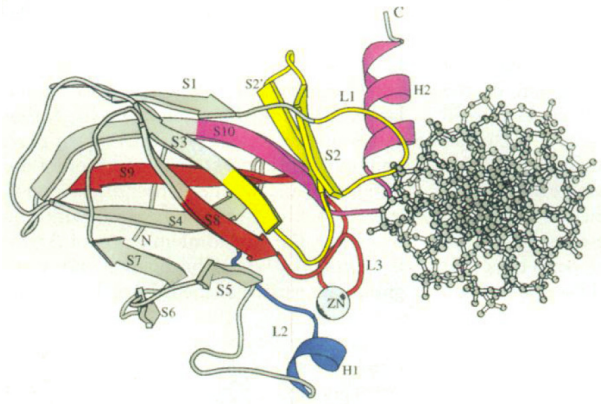

Figure 1. A schematic of the p53/DNA complex. The axis of the DNA molecule is perpendicular to the plane of the page.[25]

The domain we focused on is the DNA binding core. According to the IARC TP53 database, more than 80% of p53 mutations are found within this region.[29] Studies using

proteolytic digestion have shown that the core domain is independently folded from the other regions of p53 and also that p53 contains a Zn^{2+} atom.[30] The crystal structure of the DNA binding domain complexed with DNA, residues 93-292, was first done by Cho *et al*.[25] The core itself consists of two antiparallel β sheets, with one sheet containing four strands and the other containing five. The two sheets pack side-by-side, forming a β sandwich Greek Key super secondary structure that is a very compact, hydrophobic core. At the site of DNA contact three loops and an α helix are important for DNA/p53 complex formation. The L1 loop (residues 112-124) and the H2 α helix (residues 278-286) bonds with the pyrimidine bases in the consensus sequence in the wider major groove of DNA. The L3 loop (residues 236-251) forms important bonds with the more narrow minor grove of DNA within the adenine and thymine rich region of the consensus sequence. The L2 loop (residues 163-195) does not directly contact DNA but does have an important role in interacting with the L3 loop. The Zn^{2+} atom is ligated to three cystienes and a histidine, typical of many zinc-finger proteins, and has been shown to be very important in holding the tertiary structure, specifically the L3 loop, into this aforementioned structure by experiments done with chelating agents and a zinc-free p53 binding core crystal structure.[31,32]

iv. Stability and structural studies of wild-type and mutant p53/DNA complexes

Many of the experiments performed thus far attempting to quantify the stability of common core domain mutants in the literature use only the core domain, commonly residues 94-292, instead of the entire 393 residues that typify the wild-type protein. It has been demonstrated by Ang *et al.* that four common destabilizing mutants cause identical destabilization as determined by the proteins apparent melting temperatures.[22] This indicates two key proofs of principle: 1. The core domain can be used for stability studies of p53 mutants for function rescue

studies and 2. The core domain is pivotal in maintaining the overall stability of the full-length protein.

As mentioned previously, many of the mutations found in p53 are located within the core domain. The six most common mutated residues (Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282) are indeed located within the DNA-binding surface of the core domain.[29] Most core domain p53 mutations fit into two distinct categories, dependent on their location. DNA/p53 contact mutants, such as R248Q and R273H, change a residue into one that can no longer bond with DNA.[33] Structural mutations make up the second class, which are exemplified by R175H, G245S, R249S, and R282W. This second class of mutations is likely to alter the structure of the p53 core domain in a way that effects the abilities of other key residues to make contact with DNA.

The wild-type p53 binding domain has been demonstrated to be very sensitive to changes in temperature at 37 °C. During urea-induced denaturation wild-type p53 forms an aggregate at 37 °C, a calculated T_m of only 42 °C, and at 25 °C the free energy of unfolding is only 6.0 kcal•mol^{-1}.[34] This implies that even slight changes in p53 stability would likely result in universal unfolding and loss of function. Indeed, it has been shown that slight changes do cause global denaturation of common cancer mutation containing p53 core domains, as a decrease of free energy between 0.5 kcal•mol^{-1} and 2.0 kcal•mol^{-1} will strongly distort the folded state and a change of 3.0 kcal•mol^{-1} is likely to cause global denaturation and total functional loss.[35]

A stabilized p53 variant designed by semirational methods has been created by Nikolova *et al*.[36] They selected 22 homologous proteins and identified candidates for point mutation substitutions along with incorporating another mutation, N239Y, that had been reported to be a second site suppressor mutation for the mutation G245S. After utilizing four mutations that

increased thermostability, found by consensus analysis, one stabilized p53 protein was created with the mutations M133L, V203A, N239Y, and N268D. The T_m of the quadruple mutant was calculated to be 47.2 °C, a significant improvement over the wild-type p53 T_m of 42 °C. The crystal structure of the quadruple stabilized mutant has shown to create additional hydrogen bonds with the consensus DNA binding sequence, thus stabilizing it.[37] The stabilized mutant has been implicated in use for gene therapy but also suggests that p53 can be stabilized by creating additional linkages within the DNA/p53 complex. The crystal structures of many destabilized mutants have been solved using the quadruple stabilized core domain protein.[33]

From the change of free energy and quadruple stabilized mutant experiments a method of functional rescue for p53 mutant destabilizing mutants can be derived. If more linkages can be created between the p53 binding domain and the DNA consensus sequence, then it is plausible that the p53 mutant molecule could be stabilized, allowing for transcription of important p53 controlled regulatory genes. While this has been done by creating second site suppressor mutations to create the quad mutant, it is also plausible that this could be done by the addition of a small molecule that is either bound to p53 and assisted in molecule binding or by binding both p53 and the DNA sequence.[36]

v. The negative p53/consensus binding domain screen

To date, have been limited developed screens that can detect if p53 is stable enough to form a p53/DNA complex. A screen of this type would be greatly helpful in identifying molecules that direct help make p53 a better co-partner of the p53/DNA consensus binding domain dimer. Brinda Ramasubramanian, a graduate student in Magliery lab, has constructed a plasmid based system of p53/consensus binding domain complex formation. Using a pGFPUV vector (figure 2), a p53 consensus binding

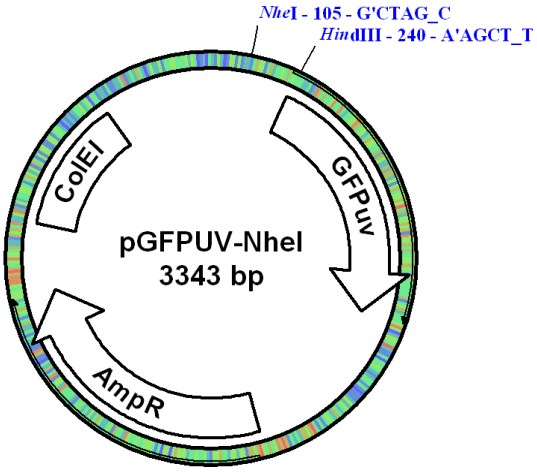

Figure 2. The pGFPuv plasmid with the restriction sites used for cloning highlighted.

domain was cloned in between a HindIII and NheI restriction site, effectively replacing the lactose operator in the parental plasmid.[38] The idea behind the screening vector is that p53 variants that are stable will bind to the consensus binding domain tightly and GFP production will be inhibited. P53 variants that are destabilized will not bind to the domain tightly and fluorescence will occur.

As a proof of principle, the stabilized quadruple mutant p53 variant, described here previously, has been shown to inhibit fluorescence production in this system while wild type p53 does not. The reason wild type p53 likely does not pass the screen is that p53 has been shown to be destabilized even at body temperature. This means that the wild type is too unstable to bind the consensus domain.

B. BRCA1

i. The BRCA1 protein significance in cancer development

Breast Cancer Susceptibility Protein 1 (BRCA1) was linked to cancer development by a genetic analysis of breast cancer in certain families that located common mutations in breast tumors to the 17q21 chromosome in 1990.[39] Statistical analysis by Ford et. al, showed that familial breast cancer predisposition is correlated to mutations within BRCA1. They have calculated a 100% percent lifetime risk of either ovarian or breast cancer in those who carry BRCA1 germline mutations.[40] Current chemotherapeutics used against breast cancer have mixed results in use with patients carrying BRCA1 mutations.[41]

Tumor cell lines that are BRCA1 deficient display a higher death rates after exposure to Gamma-rays than cell lines that have active wild-type BRCA1, indicating an inability to cope with stress that causes DNA damage. [42] A conditional mutant mouse model of BRCA1 develops mammary tumors that show very similar chromosomal instabilities to human breast cancer, indicating a major role for BRCA1 in breast cancer.[43] It has been postulated that BRCA1 deficient cell lines cannot begin cancer formation unless oncogenic mutations occur in other important tumor growth inhibiting proteins. The loss of BRCA1 in mouse embryos leads to lethality in the presence of mice positive for p53, while the haploid loss of p53 in the same model leads to rescue from embryonic lethality. However, mouse embryos deficient in BRCA1 and haploid deficient in p53 develop mammary tumors at an abnormally high rate and eventually lose the remaining wild-type allele of p53.[44,45]

An important binding partner of BRCA1, BRCA1-associated ring domain protein (BARD1), has been implicated in tumor suppression upon complex formation with BRCA1. The loss of BARD1 results in embryonic lethality and genomic instability.[46] Conditional mutants of

BARD1 have similar levels of chromosomal instability as BRCA1 conditional mutants.[47] This implies both proteins are needed for BRCA1 to act as a tumor suppressor *in vivo*.

ii. The role of BRCA1 in vivo

BRCA1 is implicated in many of the responses to DNA damage. Most notably, BRCA1 has been shown to play a major role in chromosomal stability.[48] BRCA1 associates with Rad50, the homolog of the bacterial RecA protein, in the endogenous response to DNA double strand breaks.[49] BRCA1 has been associated with many transcription factors, most importantly estrogen receptor-α, STAT1, and p53.[50] BRCA1 is involved in DNA decatenation in association with topoisomerase IIα during the S phase of mitosis.[51]

Another function BRCA1 plays in response to cellular stress is the ligase function it performs by appending ubiquitin molecules onto a varying array of substrates., including autoubiquitination of the complex formed BRCA1/BARD1 association.[52] Importantly, this ubiqutination function has been shown to be important in the response to DNA damage.[53] BRCA1 has been implicated in the ubiqutination of many important proteins, including FANCD2 , a protein associated with specific types of cancer, and RNA polymerase II, as a method of degrading stalled replication forks, allowing access for repair machinery.[54,55] While BRCA1 can still act as a ubiquitin ligase as a monomer, the ubiquitin ligase activity is dramatically increased once BRCA1 complexes with BARD1.[56] Also, the stability of BRCA1 is increased upon association with BARD1.[57]

iii. The Structure of BRCA1and BARD1

BRCA1 is composed of 1,863 amino acids.[58] Currently, no crystal structures have been reported of the full-length protein. However, two important segments of BRCA1 have been thoroughly characterized to date. The C-terminal region contains two 90-100 amino acids motif repeats deemed the BRCA1 C-Terminal repeats (BRCT), amino acids 1646-1863.[59] The other region, which we focused on, is the RING finger domain and the flanking sequences contained within amino acids 1-109 of the N-Terminal of BRCA1. This region was found by Meza *et al.* to be the minimal sequence possible for complex formation of BRCA1 and BARD1.[60] Residues 23-76 compose the Really Interesting New Gene (RING) finger domain while the remaining sequences are necessary for formation of the functional domain. Many important protein-protein interactions are mediated through RING finger motifs.[61] These motifs contain two bound Zn^{2+} molecules with each atom ligated tetrahedrally, by either four cysteines or three cysteines and a histidine, in a unique cross brace system. A central α-helix links the two Zn^{2+} binding sites. The cross brace system based on zinc ligands is important for stabilization of the RING motif.[62] Residues 8-22 and 81-96 of BRCA1 are composed of antiparallel α-helices that flank and are largely held into correct conformation needed for BARD1 association by the RING domain.[63]

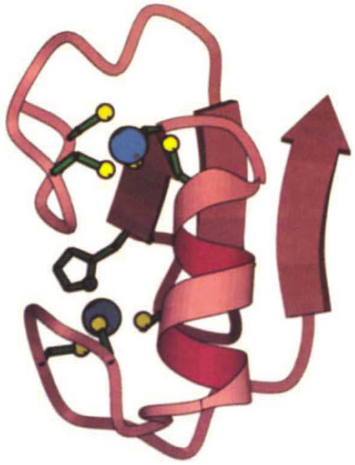

Figure 3. RING domain produced via NMR from the Equine Herpes Virus, 1-Gene 63.[62]

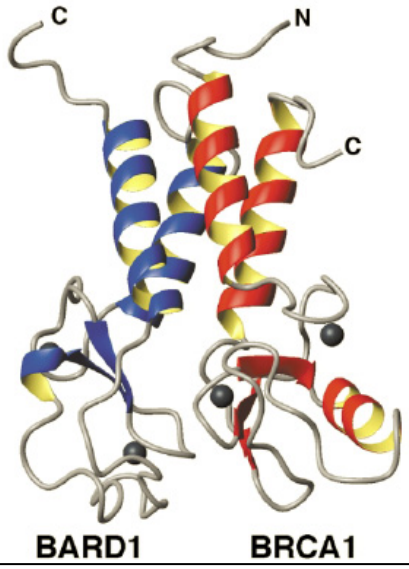

Figure 4. BRCA1/BARD1 protein complex.[63]

BARD1 is composed of 777 amino acids and first suggested to be an important binding partner of BRCA1 after being found to interact in a two-hybrid yeast screen.[64] BARD1 was found to need only residues 26-131 in order to form the BRCA1/BARD1 complex.[60] BARD1 contains a RING motif within that segment, residues 49-100. The RING residues in BRCA1 and BARD1 are almost identical albeit the RING motif in BARD1 contains five fewer amino acids, which are found as a central helix within the third and fourth pairs of Zn^{2+} ligands in BRCA1. [63] Also, both the amino terminus and carboxy terminus of the RING motif in BARD1, residues 36-48 and 101-116, are flanked by antiparallel α-helices.

iv. Mutational Studies of the BRCA1/BARD1 protein complex

Twenty percent of mutations considered clinically relevant are located within the first 100 amino acids of BRCA1, a region containing the RING domain. (Breast Cancer Information Core Website) Two general classes of mutations are commonly seen in the BRCA RING domain and flanking antiparallel α-helices. The first class consists of mutations that affect the Zn^{2+} residues within the RING-finger motif. The known cancer mutation C61G, which alters a conserved site II Zn^{2+} binding ligand in BRCA1, results in proteolytic susceptibility indicating global unfolding when compared to the proteolytic degradation resistance of the wild-type BRCA1 RING motif.[65] While some mutations within the RING domain totally inhibit the formation of the BRCA1/BARD1 complex others commonly seen in this region do not, yet the

latter mutations still inhibit the E3 ubiquitin ligase function of the complex.[66,67] This could either be from the result of a less stable BRCA1/BARD1 complex or the rearrangement of the cleft needed for E3 ligase function.[68]

The second class of mutations involves alterations within the dimerization interface of the antiparallel α-helices. Several common mutations seen in patients line this region.[63] Mutational studies done on BARD1 have shown that changing the strongly hyrdrophobic amino acids that line the dimerization interface to hydrophilic amino acids will inhibit formation of the complex.[69]

There are no known mutations seen in patients within the RING motif and antiparallel α-helices or BARD1.[70] It is unknown why mutations similar to those found in BRCA1 are not found in BARD1. However, the fact that only the RING motif of BRCA1 plays a role in E3 ligase activity probably plays a role in this strange finding.[68]

v. The Split GFP-screen

In order to directly assess the BRCA1/BARD1 dimer, some form of functional assay is needed to report if the complex has formed. Screens that detect protein-protein interactions have been developed and utilized with varying success for many different protein-protein interactions. Yeast two-hybrid systems, fluorescence resonance energy transfer (FRET), tandem affinity purification (TAP) , split protein complementation assays, and protein microarrays are commonly used methods of detecting protein-protein interactions *in vivo* and *in vitro*.[71,72] Among those, split protein complementation has risen to become a very powerful tool in discovering and detecting protein-protein interactions.

The paradigm for a split complementation based on green fluorescent protein (GFP) was first proposed by Ghosh *et al.*[73] The actual assay was developed and tested by Magliery *et al.*[74] The split-GFP screen is fluorescent when each half of the split GFP protein is bound to two copartners of an active protein-protein complex. Importantly, GFP does not fluoresce unless a stable complex is formed.[67]

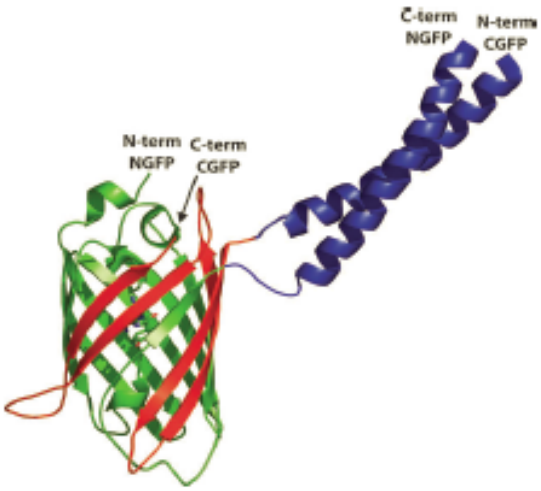

Figure 5. The Split-GFP reassembly protein. The green segment is the N-terminal half and the red segment is the C-terminal half.[67]

The assay has been used to identify mutations that inhibit formation of BRCA1/BARD1 complexes. Pairing the split-GFP assay with libraries of drug-like molecules is a potential way to find drug leads that ameliorate the binding problems caused by BRCA1 mutations found in common cancers can present.

C. Small Molecules Used As Chemical Chaperones

i. Previous Work Utilizing Small Molecules to Rescue Misfolding Mutations

A great body of evidence has mounted suggesting many protein-protein interactions are governed not by large numbers of weak interactions over a large area but a small number of important residues in small patches.[75] This idea can be applied to the use of small molecules as a method of rescuing protein-protein binding problems. An ideal location of binding was called a "hot spot" by Clackson and Wells. Hot spots have roughly the size of small organic molecules, opening the possibility of a very small molecule that could strongly bind to these hot spots in order help mediate protein-protein interactions. This discovery has led to small molecules that function in this way: Nutlin, an inhibitor of MDM2 and an inhibitor of Bcl-2 family proteins.[76,77]

Mutations found within the core domain of p53 are considered drugable by small molecules because destabilized mutants have melting temperatures below body temperature, giving these unstable proteins incredibly short half-lives.[78] If a molecule could selectively bind to the wild-type conformation of p53 then the half-life of p53 *in vivo* would greatly increase. CDB3 was the first molecule found to stabilize misfolded p53.[79] Following the discovery of CDB3, PRIMA-1, a different molecule, was found to also stabilize p53 although it is not entirely clear how PRIMA-1 does this.[80] However, the best example of a small molecule utilizing a Clackson and Well "hot spot" is exemplified by PhiKan083.[81] PhiKan083, which was found via *In Silico* screening, fits within a cleft in the

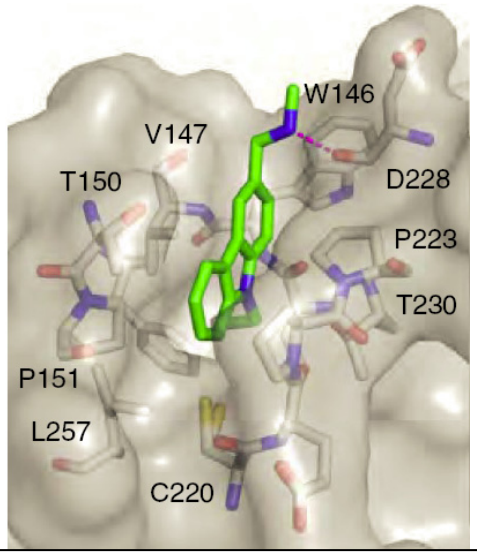

Figure 6. PhiKan083 within the cleft created by the Y220C mutation.[81]

Y220C p53 core domain mutant and has been shown to stabilize that variant of p53. In fact, the cavities that exist on the surface of Y220C have recently been characterized.[82] It is very evident that small molecules have great potential as modulators of protein function.

ii. SICLOPPS Libraries as a Method of Complex Formation Rescue

In general, peptides and small proteins have been shown to be good tools for modulating enzymes and other proteins.[83] However, the use of peptides as drugs is limited due to proteolysis caused by many of the proteolytic enzymes that exist in human blood and organs.[84] Also, linear peptides can have low specificities to binding sites due to the need to overcome large conformational entropy loss in order to adopt a single major conformation.[85] Cyclization of peptides has been shown to be a good method of circumventing both of these pitfalls. Introducing a constraint to peptides reduces the number of conformations possible, thus lowering the entropic loss needed to be overcome for binding to occur. This method has been successfully employed in developing a cyclic peptide that binds with much greater affinity to Streptavidin compared to the linearized peptide.[86] Also, this makes it less likely the peptide will take on a conformation that is able to fit within the cleavage sites of proteases.

Libraries made by the Split Intein-mediated Circular Ligation of Peptides and Proteins (SICLOPPS) method are good candidates for modulators of protein function.[87] Because the libraries are genetically encoded and produced *in vivo* the molecules do not need to cross the cellular membrane, a confounding factor that often hinders *in vivo* screening of synthetic molecules. Also, library members that are toxic will kill the cells that contain it, eliminating them from further being screened as possible drug scaffolds. However, the library is similar to synthetically derived libraries because of the wide variance in amino acids used at each position. A library containing five variable residues has over 3.2×10^6 different library members, owing to

a possible 20 amino acids at each position. The library size makes the library more like a synthetic library while still being produced using cellular machinery. This also opens up the possibility for functional screening for molecules based on phenotypic selection within the cells. Because of the ease of screening many bacteria at one time, this opens an advantageous way of discovering possible peptide modulators of protein function.

This protein trans-splicing mechanism is derived from the *dnaE* gene of *Synechocystis* species PCC6803.[88] The *dnaE* gene transcript is made of two distinct segments. After trans-splicing the extein segments form one long protein fragment while the intein segments are spliced out. In the methodology developed by Benkovic, The C-intein segment is placed at the N-terminus of the sequence to be cyclized and the N-intein is placed at the C-terminus of the sequence. Once the gene is translated the N-intein and C-intein segments fold and form the active intein complex. Once this occurs a sulfhydryl side chain, which lies within the C-terminal intein as decribed by Scott *et al.*, undergoes nucleophilic attack of the carbonyl carbon of the amino acid directly beside the sequence to be cyclized. This forms a thioester lariat intermediate. Following this, an asparagine residue cyclizes to form a lactone. The intein then self-liberates, via an isomerization reaction, causing the sequence to form a lactam ring that is backbone cyclized from the N-terminus to the C-terminus of the sequence. [87,89]

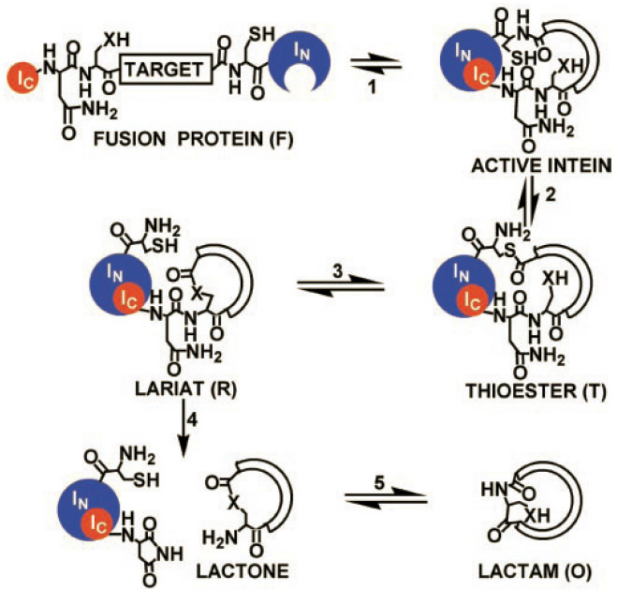

Figure 7. Circular Ligation Mechanism.[87]

There are limitations on what residues can be placed at the positions within the sequence to be cyclized beside the N-intein and C-intein segments.[90] It appears the position directly beside the C-Intein, in the N-terminus of the sequence, can tolerate only a limited number of residues. Two different groups have shown that a cysteine must be placed immediately beside the sequence to be cyclized.[90,91] The position on the C-terminus beside the N-intein segment can seemingly tolerate any residue, as this residue does not actively participate in the reaction mechanism. From the same studies, it appears there is limited selection for specific residues at other positions, meaning that library members theoretically should be diverse in the residues utilized.

SICLOPPS libraries have been used to discover possible drug scaffolds with success. SICLOPPS libraries have been used to find molecules that inhibit Dam Methyltransferase, molecules that decrease α-synnuclein toxicity in Parkinson's disease yeast models, and inhibitors of ribonucleotide reductase.[91,92,93] Given these past success, SICLOPPS libraries hold great promise for modulating other protein-ligand interactions which could lead to new drug scaffolds.

Chapter 2 - Methods

A. Materials

Most chemicals were purchased from Sigma, Aldrich, Fisher, and American Bioanalytical. Ampicillin, kanamycin, and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were bought from Research Products International and American Bioanalytical. 1000X stock solutions of Ampicillin, Kanamycin, and IPTG were made as follows; 100 mg mL^{-1} ampicillin, 35 mg mL^{-1} kanamycin, and 100 mM IPTG. The concentration of IPTG, however, depended on the final concentration needed as has been specified when used differently. L-Arabinose was purchased from Benton Dickson and made in 19.6%(w/v) solutions. Ampicillin, kanamycin, IPTG, and arabinose was sterile filtered using 0.2 μm syringe filters (Milipore). Nucleotide Triphosphates were purchased from American Bioanalytical individually at 100 mM concentrations and mixed equimolar to 10mM stock dNTP solutions. Restriction enzymes, Ligases, and electrophoretic molecular weight standards were purchased from New England Biolabs. Herculase polymerase and associated buffers were purchased from Stratagene. Oligonucleotides were purchased from Sigma-Genosys and resuspended in purified water to 100 μM solutions. The *E. coli* DH10B cloning strain and the *E. coli* BL21(DE3) expression strain were both gifted from the Lynne Regan lab. The pET28b-Npulc/In-SICLOPPS vector was a gift from the Stephen J. Benkovic Lab. The pGFPUV vector was gifted from Peter Schultz. Water used for molecular biology, specifically for making stock solutions of various chemicals, digests, ligation, and in other protocols, was purified using a Barnstead NANOpure Diamond system to 18MΩ·cm. Plasmid isolation was done using EconoSpin™ DNA spin columns from Epochlabs, using a miniprep vacuum manifold from Qiagen and buffers made within the lab according to recipes provided by Epochlabs.

PCR was performed using a C1000™ Thermal cycler (BioRad) with a heated lid on. Concentration of DNA was done using a Savant SC110 speed-vac with low or medium heat. Electrophoresis was performed in a 10 cm horizontal gel apparatus using 1% agarose gels. A BioRad PowerPac Basic was used as a power supply for all electrophoresis done. Electroporation was done using a BioRad Micropulser in 0.2 cm electroporation cuvettes purchased from USA Scientific. Cell culture was typically carried out in 50 mL Falcon tubes made by Becton Dickson or 14 mL polystyrene Becton Dickson Falcon tubes (for recovery of cells) or 14 mL polypropylene Becton Dickson Falcon tubes (for general growth). Centrifugation was done in an Eppendorf 5415 D centrifuge (for small volumes typically <3 mL), an Eppendorf 5810 R (for 5-50 mL volumes), or a Sorvall RC-6 centrifuge (for high speed and large volumes >50 mL). Cell density measurements were determined by Agilent 8453 UV-visible spectrophotometer. Pictures of plates were done using a Canon Powershot A550 camera. Fluorescence was determined by visual inspection of plates on a High Performance UV Transilluminator (UVP) and recorded using Kodak software.

B. General Protocols

All enzymatic reactions were carried out using conditions suggested by the reagent supplier, with deviations from this if needed. The standard PCR reaction was a 25 µL or 50 µL reaction volume using Herculase II (Stratagene) in 5X Herc II buffer (provided with Herculase), 4 uM primers, 250 µM dNTPS, 0.5 unit or Herculase, and 0.02 mM uL^{-1} of template DNA. Typically, each PCR was done using thirty cycles of denaturation, annealing, and extension. A typical first denaturation was for two minutes at 95 °C with subsequent denaturation being thirty seconds also at 95 °C. Annealing was done for thirty seconds with a temperature dependent on primer specific annealing temperatures. For most PCR reactions the annealing temperature was

60 °C or calculated using five to ten degrees below the lowest annealing temperatures between the two primers. Annealing temperatures were derived from the program OLIGOTECH. Extensions was done at 72 °C and for a time determined by the length of the amplified region desired, generally calculated by one minutes per 1 kilobase of product. A final extension temperature was done at 72 °C for 5-8 times longer than the extensions done in the previous cycles. All PCR products were analyzed using agarose gel electrophoresis to determine if the desired product was achieved. The restriction enzyme DpnI, which only cuts methylated template DNA, was added at 0.2 units mL^{-1} of PCR reaction after all SICLOPPS library insert amplifications. PCR products were purified using the QiaQuick PCR purification protocol provided by Qiagen, with the only exception being a different buffer instead of PB (I used PB1 as described by Epochlabs).

Restriction digests were done as according to the protocols given by New England Biolabs. Ligations were typically done as according to New England Biolabs protocols, with the amount of DNA depending on if a single clone or complete library was desired. Phenol-Chloroform-isoamyl alcohol/Chloroform-isoamyl alcohol followed by ethanol precipitation and/or agarose gel purification with a QG cleanup (as described by Qiagen) was done in order to clean up DNA fragments produced after restriction digest.

Fresh electrocompotent cells (DH10B or BL21(DE3)) were prepared by the author after successful ligations of all libraries to ensure maximum transformation efficiency and maximal library coverage. Electrocompotent cells were prepared in 0.5 L or 1.0 L volumes of culture in 2YT liquid media. Seed cultures (1/40 dilutions of the preparation amount) were inoculated from single colonies of the strain desired and grown to saturation (12 to 14 hours) at 37 °C. The seed was then used to inoculate the preparation amount and the preparation was grown at 37 °C to an

absorbance of 0.5 O.D. at 600 nm and then vigorously shaken in ice water for five minutes. The culture was always kept in ice throughout the following process. The preparation was then centrifuged in the Sorvall centrifuge at 7,000 RPM for five minutes, the supernatant was pulled off, and the pellet was resuspended in 4 mL of ice cold autoclaved 10% glycerol (v/v). A second identical centrifugation, resuspension and centrifugation was preformed. After this the supernatant was pulled off and the pellet was resuspended in small amounts of 10% glycerol (v/v) and aliquoted in 100 μL amounts and then flash frozen in dry ice. All electrocompotent cells were stored at -80 °C for future use. All electrocompotent cells quality controlled by electroporating an aliquot of the preparation, resuspending the electroporated cells in 1 mL of 2YT, recovery with vigorous shaking at 37 °C for one hour, and then plating 200 μL on separate Luria-Bertani(LB)/ampicillin and LB/kanamycin plates. If no cells grew after 16 hours of incubation at 37°C then those cells were deemed contamination free.

Electroporation was done by mixing 1 μL (10^3 dilution for single variant plasmids or undiluted for libraries) miniprepped, circular DNA in 40 μL electrocompotent cells (for single variants) or 100 μL electrocompotent cells (for libraries, in order to ensure maximal diversity retention). Immediately after electroporation, the cells were resuspended in 1 mL of 2YT and recovered at 37 °C for one hour. The culture was then spread on LB plates with the desired antibiotic resistance.

The transformation efficiency of all libraries after transformation into electrocompotent cells was quantified by pouring all quenched, electroporated cells into 1 L of 2YT, recovery for one hour, and then plating 10^3, 10^4, and 10^5 dilutions on LB plates with the required antibiotic resistance. After 12 hours of incubation at 37 °C, the numbers of colonies on the plates were

counted and a back calculation was performed in order to make sure all libraries were represented ten fold over the size of the given library.

All cells desired to be saved for long periods of time were glycerol stocked by adding 0.5 mL of 50% glycerol (v/v) to 1 mL of saturated culture in 2YT grown with the associated antibiotic. The mixture was then flash frozen in dry ice and then kept in -80 °C for long term storage.

DNA sequencing was performed by Genewiz. According to their protocol, each sequencing reaction contained the following components; 6 µL of plasmid miniprep, 4 µL of 2mM primer, and 2 µL of water. For sequences greater than 800 base pairs, two primers were used, a forward and a reverse to ensure coverage. All alignments were done using ClustalX2.

All plates made by the authors hands, or fellow lab members, and were allowed to dry for 24 hours at room temperature before being used. All screening plates that contained arabinose and/or IPTG were used within 36 hours of being made.

C. P53 methods

i. Cloning and glycerol stocking of the SICLOPPS Library

The SICLOPPS library was a given to us from Charles Scott, Thomas Jefferson Medical Center. The library was genetically encoded within a pET-28b vector (Novagene). The DnaE intein was cloned between a NcoI and XhoI site. The NcoI site was destroyed. The variable region was cloned in between the split inteins using an AflII and MfeI site. The library,

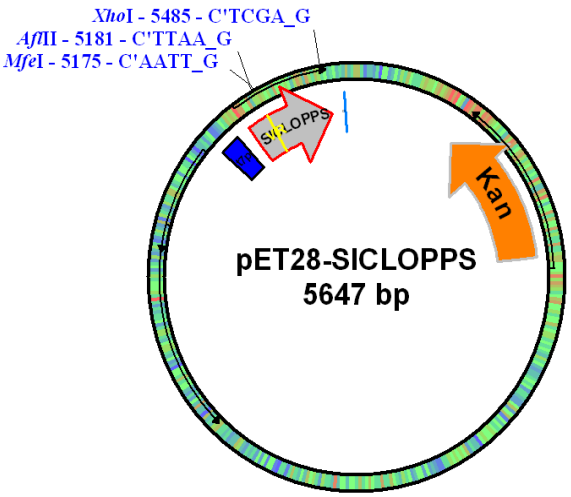

Figure 8. pET28-SICLOPPS plasmid.

encoded by the region between the AflII and MfeI sites, contains cysteine that is constant in every SICLOPPS molecule and four variable positions. All SICLOPPS molecules theoretically contain five amino acids by this design. According to this, the maximum number of unique SICLOPPS molecules is 160,000.

We were given dehydrated plasmid which was resuspended in water and then electroporated into fresh DH10B produced as described above and glycerol stocked for future use.

ii. Cloning of the SICLOPPS library into the pGFPUVbd1 vector

Primers were designed to append an AatII restriction site to the 5' end of the SICLOPPS library and a SpeI site to the 3' end of the library. The following primers were used to amplify the library using the Pet28-SICLOPPS vector as a template, using the PCR protocol described previously.

Figure 9. pGFPuvbd1/SICOPPS cloning primers

Primer Name	Primer Sequence (5' to 3')
SICLOPPS-SpeI	AATAATAATACTAGTTAATCGCCGCGACAATTTGC
SICLOPPS-AatII	ATTATTATTGACGTCTCAGTGGTGGTGGTGGTGGTG

After PCR amplification, DpnI digest, and Qiagen PCR cleanup (all as previously described), the amplified fragment was double digested using AatII/SpeI and then purified using agarose gel extraction followed by a QC cleanup.

At the same time, the pGFPuvbd1 vector, created by Brinda Ramasubramanian, a graduate student in Magliery lab, was double digested using restriction enzymes that are cut specifically at unique sites within the pGFPuvbd1 vector and also within sites appended to the ends of the SICLOPPS library insert fragment. The pGFPuvbd1 vector was double digested, with a phenol-chloroform-isoamyl alcohol/chloroform-isoamyl alcohol followed by ethanol

precipitation step in between each digest. The double digested pGFPuvbd1 vector was then agarose gel extracted followed by a QC clean-up.

The cut and purified SICLOPPS insert fragment and the pGFPuvbd1 vector were then concentrated using the Savant SC110 speed vac. Two ligations were performed, a control pGFPuvbd1 background assessment ligation and a ligation of the pGFPuvbd1 vector and the SICLOPPS insert, hereon deemed the "combined" ligation. A 20 µl reaction volume was used for both ligations, with the following components; 2 µl of 1X T4 ligase buffer, 0.5 µl of T4 ligation (~300 ng), and water to bring to the total reaction volume desired. Ligations were done at 16 °C for 24 hours. After ligation, both the control and the combined ligation were double digested with EagI and BsiWI restriction enzymes in order to linearize any background parental pGFPuvbd1 vector still present in the ligations. This preparation was electroporated into DH10B and 100 µL of those cells was plated to enumerate background contamination and transformation efficiency on LB/ampicillin plates. The CFU for both plates were analyzed in order to check background levels and transformation efficiency.

iii. Purity Assessment of the pGFPuvbd1/SICLOPPS library

A saturated culture of the DH10B/pGFPuvbd1-SICLOPPS library was grown and miniprepped. The plasmid DNA was then double digested using the cloning sites (AatII/SpeI).

Purity of individual SICLOPPS clones was assessed by plating 150 µL of saturated plasmid contained DH10B, diluted by a factor of 10^4, on LB/ampicillin plates and picking ten random variants. These variants were miniprepped and digested using the cloning sites (AatII/SpeI).

Three of the ten individual clones were sequenced using the following primers (reverse and forward).

Figure 10. pGFPuvbd1/SICOPPS sequencing primers

Primer Name	Primer Sequence (5' to 3')
p53-SICLOPPS Forward	GAATTCCAACTGAGCGCC
p53-SICLOPPS Reverse	GAAAAATAAACAAATAGG

iv. Optimization of the p53WT screen in BL21(DE3)

Brinda Ramasubramanian created the screen and optimized it for the DH10B strain. The screen needed to be optimized for usage of the BL21(DE3) expression strain. In order to optimize fluorescence of the p53 binding screen a positive control , the quadruple stabilized mutant p53, and a negative control, p53 wild type(WT), were separately co-transformed along with the pGFPuvbd1 plasmid into BL21(DE3).

Arabinose concentration was first optimized at different temperatures and times. Then IPTG concentration was optimized in the conditions deemed best for the Arabinose concentration. The following concentrations of arabinose and IPTG and also time and temperature variations were used to optimize conditions for screening:
Arabinose: 0.01%, 0.0075%, 0.005%, 0.0025%, 0.001%, 0.00075%, 0.0005%, 0.00025%
IPTG: 10 mM, 1 mM, 0.5 mM, 0.1 mM, 0.05 mM, 0.01 mM; Temperature: 30°C, 37°C; Time: 12 hours, 18 hours, 36 hours, 48 hours.

v. Assessment of SICLOPPS molecule affect on the p53 screen

To access if SICLOPPS molecules had an effect on the p53 screen two experiments were set up: DH10B strain with the pGFPuvbd1-SICLOPPS plasmid on an LB/ampicillin plates and BL21(DE3) strain with the pGFPuvbd1-SICLOPPS plasmid grown on LB/ampicilin-0.1 mM IPTG. These were incubated for 16 hours at 37 °C.

vi. Reversion of the SICLOPPS library with pGFPuvbd1

The method for cloning the reversion of the SICLOPPS library is identical to that used to originally clone the library into the pGFPuvbd1 plasmid. The only different is that the sites of the AatII and SpeI sites on the primers have been switched.

Figure 11. pGFPuvbd1/SICOPPS reversion primers.

Primer Name	Primer Sequence (5' to 3')
Bd1GFP-SCI-AaTII	AATAATAATGACGTCTAATCGCCGCGACAATTTGC
Bd1GFP-SCI-SpeI	ATTATTATTACTAGTTCAGTGGTGGTGGTGGTGGTG

vi. Selection and purification of SICLOPPS peptides

A saturated culture of 150 µL BL21/pGFPuvbd1-SICLOPPS , diluted by a factor of 10^3, was plated on an LB/ampicilin-0.1 mM IPTG and grown for 16 hours at 37 °C. Eighteen low or non-fluorescent CFU were picked, cultured, and glycerol stocked. Four of these were expressed for SICLOPPS molecules using the following protocol. A 1 mL seed of 2YT/ampicillin was grown to saturation at 37 °C for 12 hours. A 1/40 amount of final preparation (0.625 µL) of the seed was added to 25 mL of 2YT/ampicillin and grown to between 0.6-0.8 O.D. The cultures were then induced by adding 2.5 µL of 1 M IPTG to give a final concentration of 0.1 mM IPTG. The cultures were then moved to a 28 °C shaker for 24 hours. After that time, the cells were spun down at 3,200 RPM for 25 minutes at 4 °C. The supernatant was removed and the pellets were placed in -80 °C for storage. The pellets were thawed on ice. A 1/40 (0.625 µL) amount of preparation of lyses buffer (50 mM Tris-HCL, 300 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol) (0.625 µL) was added to the pellets and the pellets were resuspended. 0.1 mL of 0.1 mm glass beads (Biospec) were added and the mixture was vortexed for thirty seconds and then

chilled on ice for two minutes, repeating five total cycles of vortexing and cooling. The mixture was then spun down at 4 °C for 30 minutes at 13,200 RPM. The supernatant was pulled off and heated to 85 °C for 15 minutes. The solution was then spun 4 °C for 30 minutes at 13,200 RPM. The supernatant was pulled off and *n*-butanol extracted. Both the aqueous and butanol layers were saved. Both layers were dried using the spin-vac.

viii. MALDI-TOF analysis of SICLOPPS variant lysates

The matrix was made by saturation of α-Cyano-4-hydroxycinnamic acid in 50% acetonitrile (v/v), 50% water, and 0.1% Trifluoroacetic acid. 2 µL of this matrix was added to 1 µL of resuspended lysates (30 µL of water was added to the dried pellet and vortexed). The samples were analyzed using a Bruker MicroFlex mass spectrometer.

D. BARD1/BRCA1 methods

i. Stuffer fragment cloning into pET11A-BARD1

A lack of two unique restriction sites within the pop out of pET11a-BARD1, a plasmid

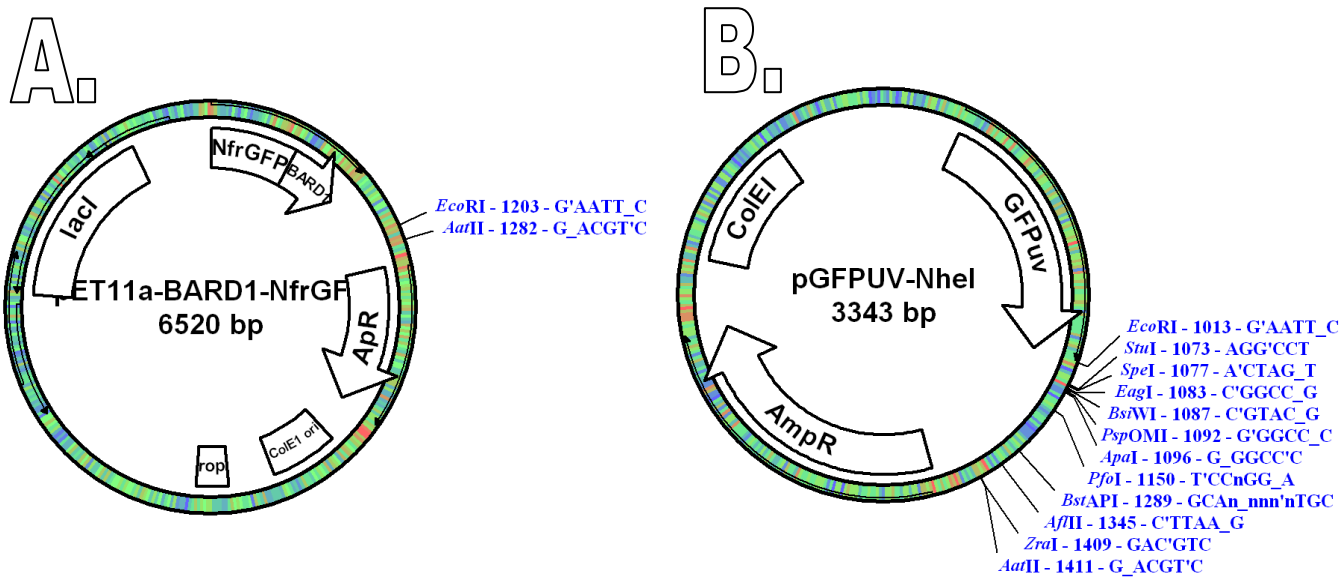

Figure 12. **A.** pET11a-BARD1 with eventual clone in sites for the SICLOPPS library
B. pGFPuvbd1 with stuffer fragment between EcoRI-HF and AatII site. Multiple background digest sites are shown in between the cloning sites.

designed by Mohosin Sarkar of the Magliery Lab, led to the need for cloning a "stuffer" fragment with the sites that would eventually be used for cloning the SICLOPPS library into pET11a-BARD1. DH10B with a pET11a-BARD1 plasmid (ampicillin resistance) and, separately, DH10B with pGFPuvbd1 was grown 12 hours in 2YT/ampicillin at 37 °C, miniprepped, and digested with EcoRI-HF and AatII. Two 5 µL ligations, a background ligation with only pET11a-BARD1 vector and a combined ligation with both pET11a-BARD1 vector and pGFPuvbd1 insert, were performed with the following ingredients; 0.5 µL of 1X ligation buffer, 0.2 µL of ligase, 3 µL of pET11A-BARD1 digest (50 ng), 1 µL of pGFPuvbd1 digest (combined ligation only, ~50 ng), and brought each ligation to 5 µL. Each ligation was done at 16 °C for 24 hours. Each ligation was then transformed into DH10B and the 100 µL of recovery was plated on

LB/ampicillin. The combined ligation plate was observed under UV light (302 nm λ) and ten non-fluorescent colonies were selected.

The ten colonies were digested with the cloning restriction enzymes and run on an analytical gel. Three of the variants were sequenced.

Figure 13. pET11a-BARD1 sequencing primers

Primer Name	Primer Sequence (5' to 3')
SICL/brd-seq-fwd1	TGCCGGCCACGATGCGTCC
SICL/brd-seq-rvr1	GGGTTCCGCGCACATTTCC

ii. Cloning of the SICLOPPS library into the pET11A-BARD1 plasmid

The pET28-SICLOPPS plasmid served as the template for the BARD1 SICLOPPS library insert. Primers (figure 12) were constructed to add an AatII restriction site to the 5' end of the insert fragment and an EcoRi-HF site to the 3' end of the insert fragment.

Figure 14. pET28-SICLOPPS/BARD1 PCR primers

Primer Name	Primer Sequence (5' to 3')
SICL/brd-pcr-fwd1	AATAATAATGAATTCACGGGGCCTGCCACCATACC
SICL/brd-pcr-rvr1	AATAATAATGACGTCATCCGGATATAGTTCCTCCTTTC

The PCR was done with Herculase as described previously. DpnI was added to the PCR reaction in order to digest away pET28-SICLOPPS parental vector. A PCR cleanup was then performed on the reaction followed by a digestion with AatII and EcoRI-HF restriction sites. The digest was then agarose gel extracted and a QC cleanup was performed to purify the fragment. The fragment was then concentrated to prepare for ligation.

pET11a-BARD1-stfr was grown in 25 mL of 2YT/ampicillin for 12 hours at 37 °C. The culture was then miniprepped and digested using AatII and EcoRI-HF. The digest was then phenol-chloroform-isoamyl alcohol/chloroform-isoamyl alcohol extracted followed by ethanol precipitated followed by a second AatII/EcoRI-HF digest. The double digested DNA was then

agarose gel extracted followed by a QC clean up. The pET11a-BARD1-stfr vector was then concentrated using a spin-vac.

Chapter 3 – Results and Discussion

A. Results Preface

The overarching goal of this project was to utilize functional screens developed within Magliery laboratory to screen for small molecules that could rescue complex formation of interactions needed to stop cancer development *in vivo*. Both screens were developed and tested by other group members in Magliery laboratory. This project concerned using applying those screens to a SICLOPPS library and visually screen for bacteria that contained possible drug leads, as indicated by fluorescence expression.

In both cases, each functional screen was constructed using two different plasmids. To implement SICLOPPS encoded genes to each respective system, the genes encoding the SICLOPPS library had to be placed within one of the plasmids. For the p53 assay, the pGFPuvbd1 plasmid, which contained the consensus binding domain, was chosen to be the plasmid for SICLOPPS addition. The reasoning for this was that this allowed for facile change of the p53 mutational variant being screened, which are placed within the other plasmid in the screen, pACBAD-p53.

For the BRCA1/BARD1 assay, SICLOPPS was chosen to be placed in the plasmid containing BARD1. This was done for a reason similar to that used to justify placement in the p53 assay. Since BARD1 does not have many mutations reported in the literature that are associated with cancer, only BRCA1 mutants need to be screened for functional rescue. The meaning of this is that the SICLOPPS library can stay in the pET11a-BARD1 plasmid without needing to be recloned to test additional mutants. To test different mutants of BRCA1, only the BRCA1 plasmid needs to be switched out, depending on the variant needing to be studied for functional recovery.

B. P53 Results

i. Production of pGFPuvb1/SICLOPPS plasmid and Purity Assessment

Constructing the pGFPuvbd1 plasmid is one part of a screening system that is designed to discover new drug scaffolds that can positively affect a protein commonly mutated in cancer, p53. The system has been designed to use two different plasmids (figure 15), each containing a different selectable resistance marker, in unison to screen large,

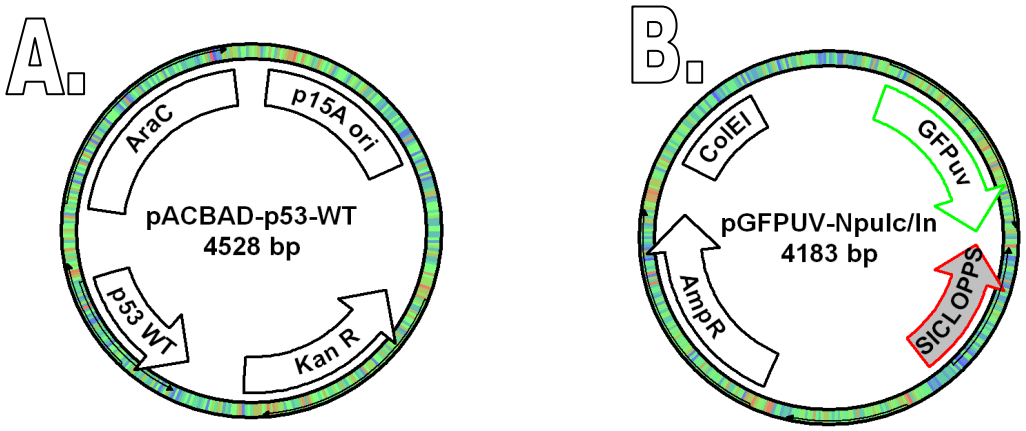

Figure 15. **A.** The kanamycin plasmid, with the various p53 variants encoded where "p53 WT" ORF is.
Figure **B.** The ampicillin plasmid, pGFPuvbd1-SICLOPPS with the split intein-SICLOPPS library cloned between AatII/SpeI

genetically encoded libraries. The kanamycin resistance plasmids, which carry various p53 variants that are under the control of an arabinose promoter, have been constructed by Brinda Ramasubramanian. The other part of the screening system, the pGFPuvbd1-SICLOPPS plasmid, are the focus of my work concerning p53. To generate the library, which needed to be placed within the pGFPuvbd1 plasmid (figure 16), pET28-SICLOPPS (a gift of the Scott lab) was grown and miniprepped. The miniprep was then used to PCR amplify the library region within the plasmid (as described in the methods).

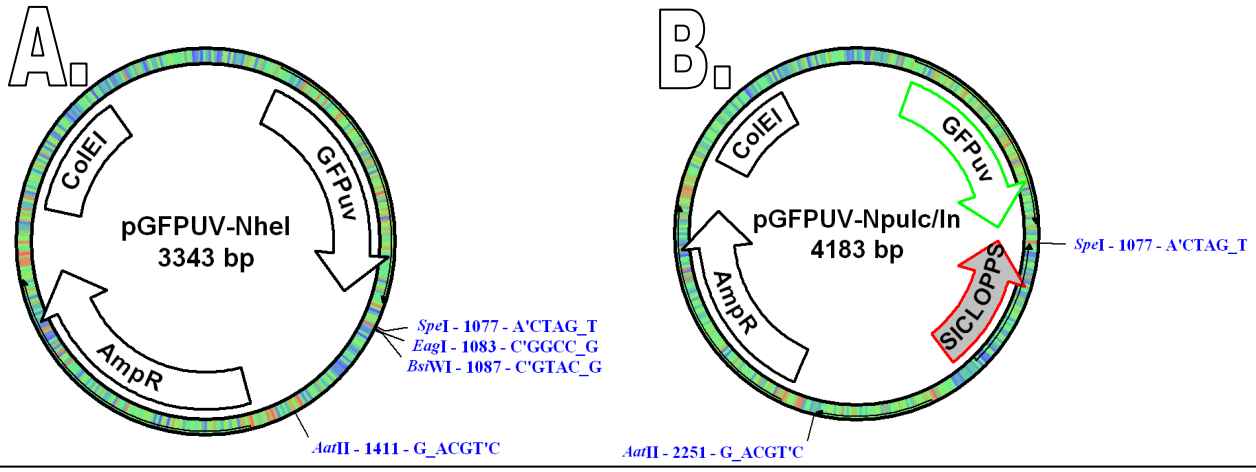

Figure 16. **A.** The vector (pGFPuvbd1) in which the SICLOPPS library was cloned into. AatII/SpeI were the sites used to clone in the library. EagI/BsiWI were used as a background digest.
Figure **B.** The pGFPuvbd1 vector with the split intein-SICLOPPS library cloned between AatII/SpeI.

The PCR product was then digested using the restriction enzymes AatII and SpeI. At the same time, DH10B cells containing the pGFPuvbd1 plasmid were grown, miniprepped, and double digested using the same restriction enzymes. The fragments were ligated, which a background digest set up in parallel with the insert and vector ligation r ensure low background of parental vector.

A major problem working with libraries using plasmid expression system is found in the vectors that the libraries are cloned in. In this case, eliminating pGFPuvbd1 vector from the library presented a problem. Because the pGFPuvbd1 vector is ampicillin resistant, just as the pGFPuvbd1-SICLOPPS library is, it is impossible to destroy background contamination by selectable marker resistance alone. To combat this, pGFPuvbd1 was digested twice and a background digest was used. However, my background ligation using only pGFPuvbd1 still had 60 CFU compared to the greater than 1,000 CFU derived from the combined pGFPuvbd1 and pET28-SICLOPPS insert ligation. While this is less than a 1:10 ratio, it is possible this could lead to problems of background noise while screening. To check and see if the background noise

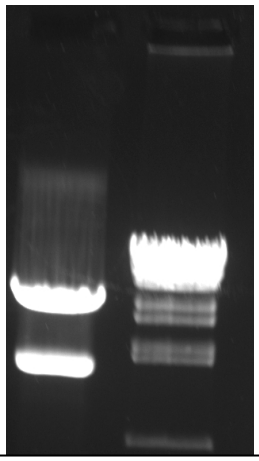

Figure 17. AatII/SpeI digest of the whole library. Ladder is 0.5 ng λ BsteII

could be better quantified, the library was miniprepped and digested with the cloning enzymes (figure 17). The gel shows a slight streak above and below the Vector band (3039 base pair (BP)) but a very distinct band that is the same size as the expected SICLOPPS library insert fragment (1077 BP). There is no noticeable band at the expected size of the pop-out band (334 BP, not shown).

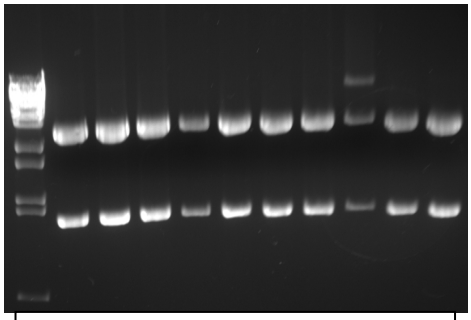

Figure 18. Digest(AatII/SpeI) of ten random colonies picked from an LB/ampicilin plate of the pGFPuvbd1-SICLOPPS library. Ladder is 0.5 ng λ BsteII digest.

The ten at random colonies picked and digested (figure 18) all show two uniform bands at the size of the expected pGFPuvbd1 vector and SICLOPPS insert. However, colony 8, or lane 9, does have what seems

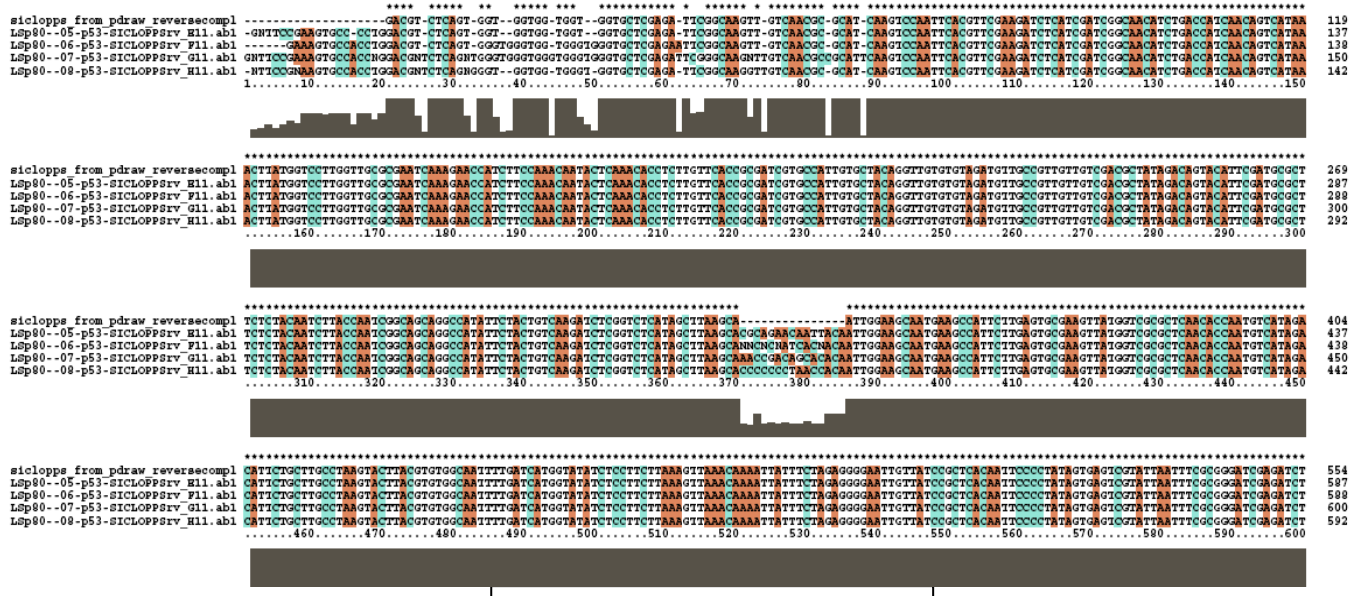

Figure 19.Reverse (3' to 5') ClustalX2 of the three random colonies. The 15 BP insertion is the library.

to be a triplicate cut. The top band, however, is impossibly large indicating it is likely this colony didn't get digested for long enough. Three of the colonies from figure 17 (lanes 2,3,9) were sent for sequencing and returned sequences without an mutations within the T7 promoter region to

the SpeI restriction site (figure 19). Only the data for reverse sequencing has been included here as the forward sequence did not cover the variable region.

ii. Optimization of the p53 screen in BL21(DE3)

Brinda Ramasubramanian constructed and optimized the maximal fluorescence levels seen in the negative and positive controls of the p53 binding screen for cells of the DH10B cloning strain. Since SICLOPPs molecules are controlled by a T7 promoter, and the DH10B strain does not have the cellular machinery needed to recognize a T7 promoter, the conditions of the screen needed to be reoptimized for the strain needed to screen the library, BL21(DE3). Other experiments done in the lab have shown that changing strains can alter the expression and fluorescence seen after plating. pGFPuvbd1 was designed with a modified lactose promoter that is placed upstream of a p53 consensus binding site. The screen Brinda has developed has two positive controls. Stabilized quadruple p53 mutant (quad) acts as the positive control, as it is hypothesized to bind the consensus binding domain and keep the modified lactose promoter from transcribing GFP, giving no fluorescence. The negative is done with wild type p53, which is hypothesized to be too unstable to bind the consensus binding domain, meaning the GFP transcription is not stopped and fluorescence is achieved. The two controls were plated side by side on various concentrations of IPTG, arabinose, time of incubations, and temperatures. The conditions chosen were 30 °C, 48 hours, 0.0075% arabinose (v/v), and 0.1 mM IPTG had the following qualities that made those conditions the best; The GFP level was highest in the p53 WT streaks, the Quad streaks showed the least amount of fluorescence, and the colonies were large and distinct. Most

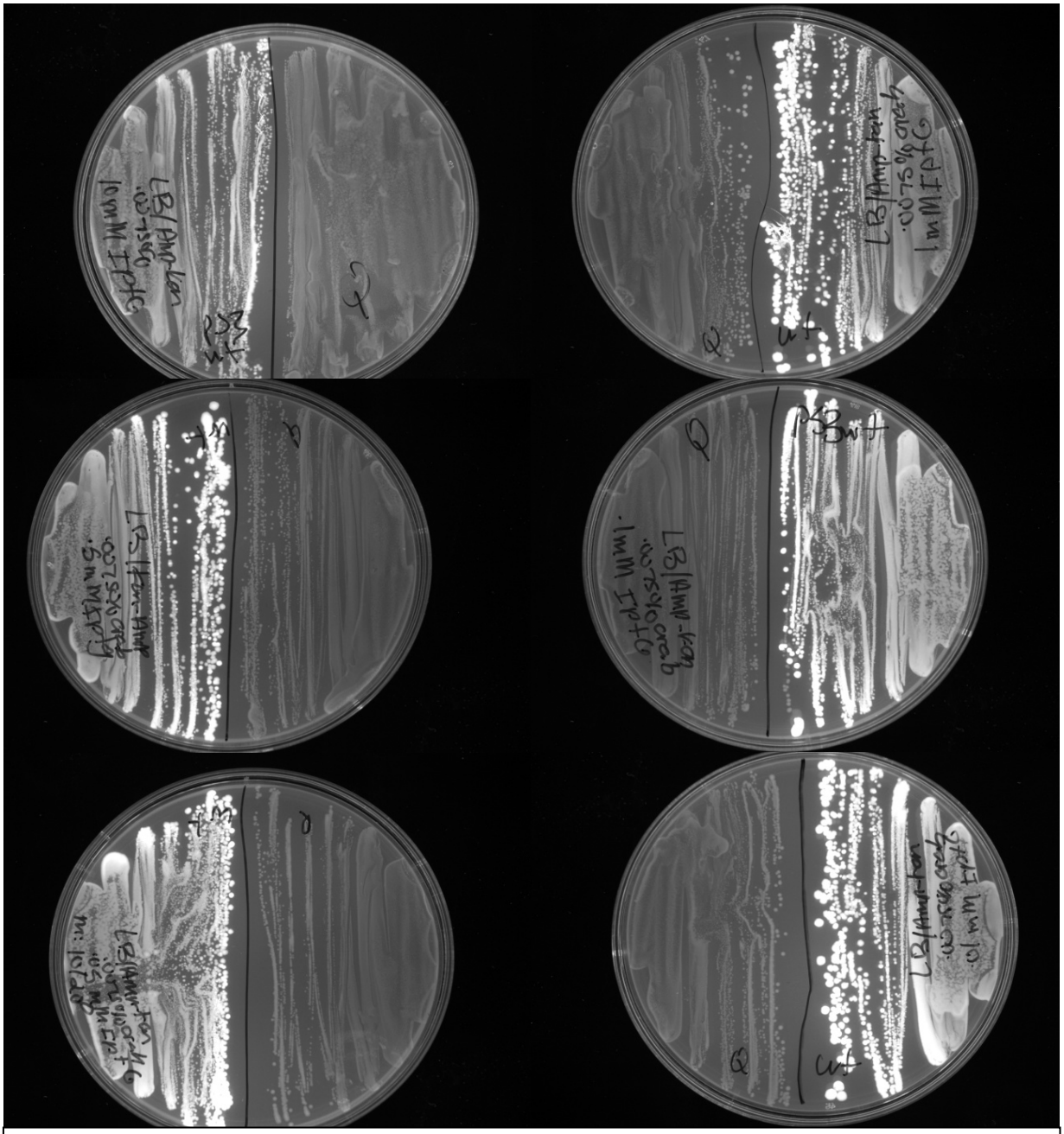

Figure 20. Optimization of IPTG levels in LB/0.0075% arabinose-Amp and various IPTG levels. From Top left to right bottom: 10mM, 1 mM, 0.5 mM, 0.1 mM, 0.5 mM, 0.1 mM. 0.1 mM was the chosen concentration.

important, the difference between the positive and negative control is the greatest, making the screen more sensitive. Interestingly, the quad variant showed fluorescence at high levels (1 mM or above) or IPTG. The modified lactose binding domain downstream of the consensus binding domain and the GFP gene should not be responsive to the amounts of IPTG added. However, it

is possible that increased transcription of GFP is due to an increased amount of T7 polymerase within the cells, since the DE3 cassette is controlled by a lactose operon.

iii. Assessment of how SICLOPPS molecules effect the p53 screen

A side-by-side comparison of BL21(DE3) cells containing the pGFPuvbd1-SICLOPPS library and DH10B cells containing the same plasmid was used to determine if expression of the SICLOPPS molecules could interfere with expression of GFP. Another group has demonstrated that SICLOPPS molecules can interfere with transcription and translation in yeast, inhibiting molecular based screens.[91] A key difference between the DH10B cells and the BL21(DE3) cells is that DH10B cells do not have cellular machinery capable of transcribing gene products governed by a T7 promoter, as the SICLOPPS molecules are. Thus, library expression is off in DH10B and turned on in BL21(DE3) when lactose or a lactose analog is supplied.

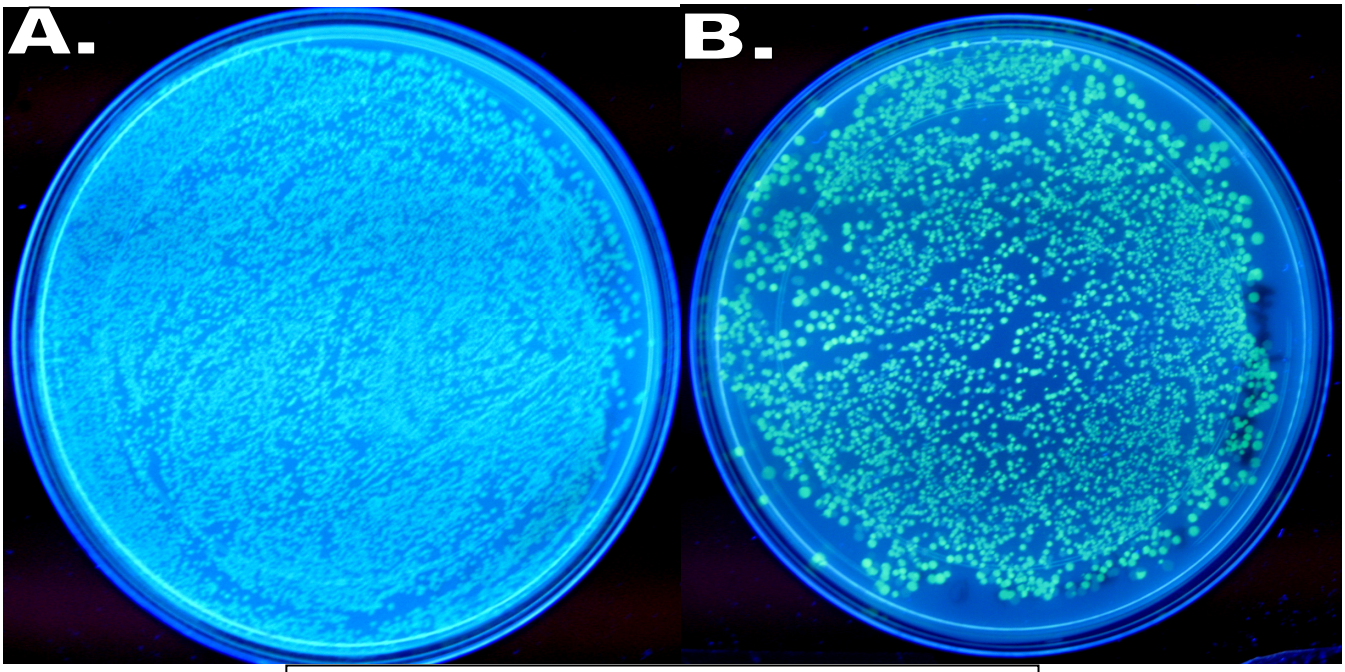

Figure 21. **A.** DH10B/ pGFPuvbd1-SICLOPPS on LB/ampiclin
B. BL21(DE3)/pGFPuvbd1-SICLOPPS on LB/ampicilin-0.1 mM IPTG.

This experiment was necessary to determine if the expression of SICLOPPS molecules could cause non-fluorescence when expressed.

Not a single non-fluorescent colony was found during visually screening of ~3,000 colonies of DH10B/pGFPuvbd1-SICLOPPS under UV light (302 nm λ). However, visual inspection of the BL21(DE3) colonies showed between a 15%-30% rate of non or low fluorescence, a very significant difference (figure 21). This indicates that many of the SICLOPPS variants, even with amino acids composition differences, have strongly negative interactions on the development of fluorescence at high rates. Inhibiting GFP maturation, whether through interference at the transcription, translation, or the protein folding levels makes screening for negative hits impossible. Figure 22 shows an enlargement of figure 21 B, displaying the fluorescent and non-fluorescent colonies in better detail.

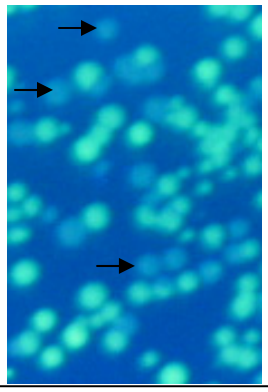

Figure 22. Enlargement of figure 21 B. Arrows are pointing to non-fluorescent colonies, for comparison.

In order to remedy this, more insight into if the SICLOPPS molecules are actually self cyclizing or if the molecules are being expressed in quantifiable amounts had to be gained. This was done by attempting to "reverse" the open read frame of the SICLOPPS library in the pGFPuvbd1 plasmid and also by attempting to purify SICLOPPS peptides.

vi. Inversion of the SICLOPPS library with pGFPuvbd1

Expression the SICLOPPS libraries caused from 15% to 30% of all variants to show no or low fluorescence. It was unknown why this was and could be due to many reasons. It was hypothesized that the lack of fluorescence could be due to SICLOPPS molecule transcription out competing the pGFPuvbd1 gene for expression, due to the inward orientation of both reading frames (figure 23 A).

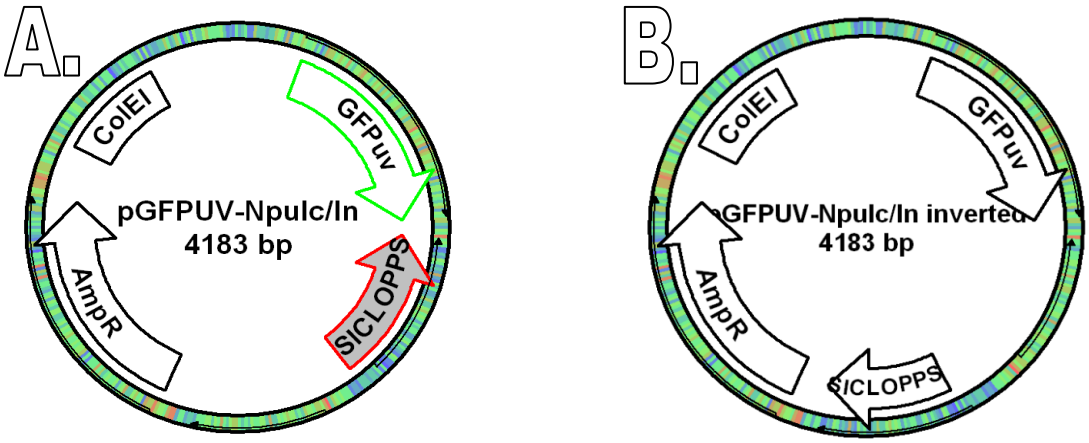

Figure 23. **A.** The vector (pGFPuvbd1-SICLOPPS) with the direction fo the SICLOPPS library that was first cloned. Figure **B.** The same parental vector as in figure 22 A except the SICLOPPS library has been turned reversed.

To test this, it was decided to reverse the SICLOPPS library to see if this allowed a greater number of all variants to fluoresce.

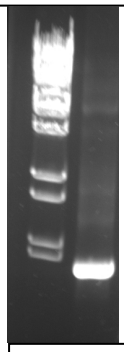

Figure 24. PCR of the reverse SICLOPPS insert(~1,100 bp). Ladder is 0.5 ng λ BsteII digest.

The cloning for this experiment has yet to be completed. Both the insert reverse SICLOPPS library and the vector have been produced and double digested (figure 24). Both fragments need to be concentrated, ligated, and then screened to see if the percentage of cells showing fluorescence has improved.

v. Selection and purification of SICLOPPS peptides

Another possibility of why SICLOPPS molecule expression resulted in failure of fluorescence could be that the SICLOPPS molecules are not completing the self-cyclization reaction. To test this, four different non-fluorescent variants were expressed as detailed in the methods second. These variants have yet to be sequenced so it is unknown what molecules they are encoded to express. Preliminary MALDI-TOF data collection has been done but more data needs to be collected.

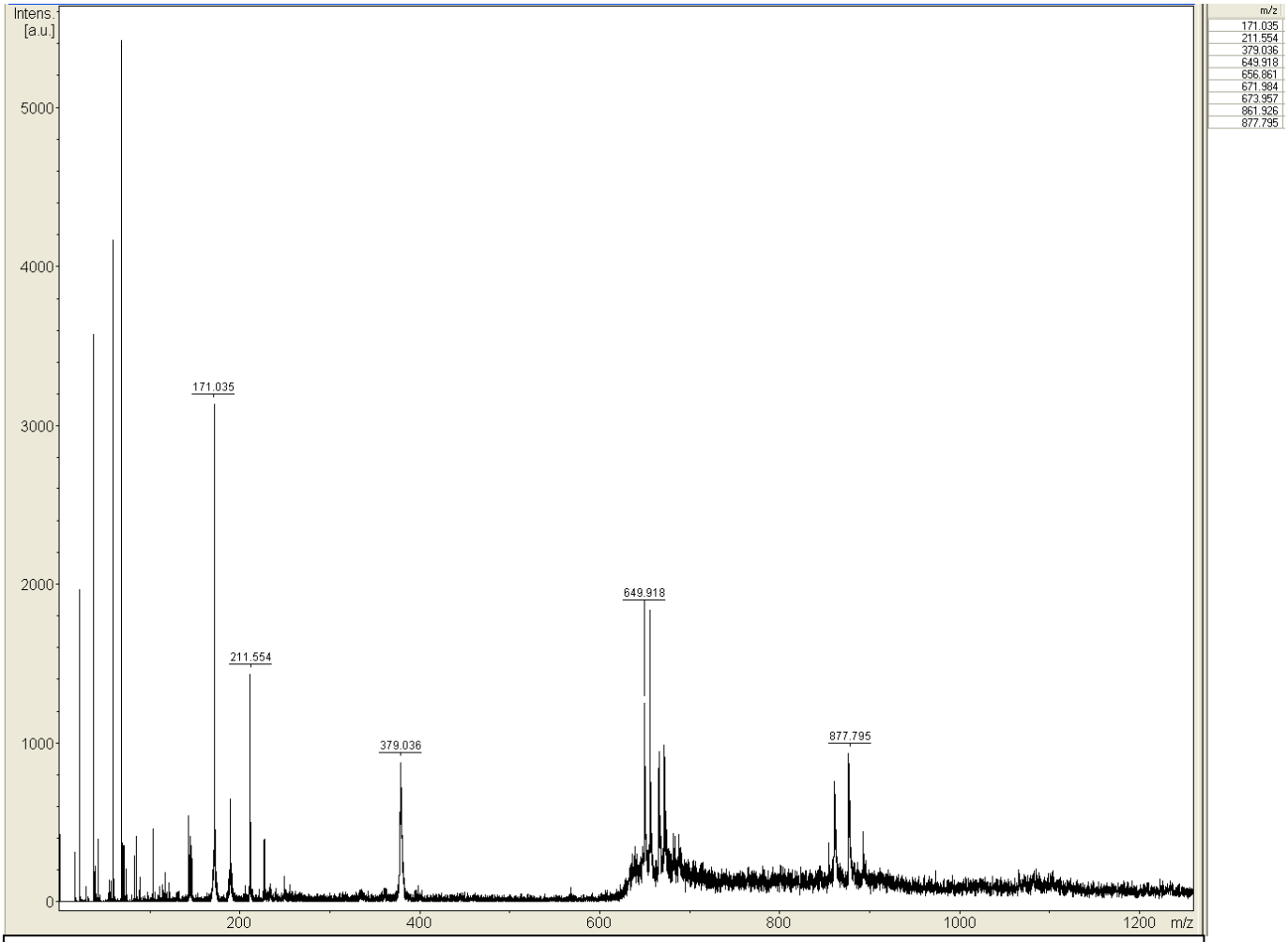

Figure 25. Preliminary MALDI-TOF data for one of the expressed variants.

C. BARD1/BRCA1 Results

i. Creation of the new parental cloning vector

The BRCA1/BARD1 screen system system, which is based on a split-GFP complementation assay, relies on a two plasmid system just as the p53/consensus binding domain system does (figure 26). Dr. Mohosin Sarkar, a previous graduate student in Magliery Lab, created the plasmids for this screen. The role of this project was to clone the SICLOPPS library into the BARD1 plasmid.

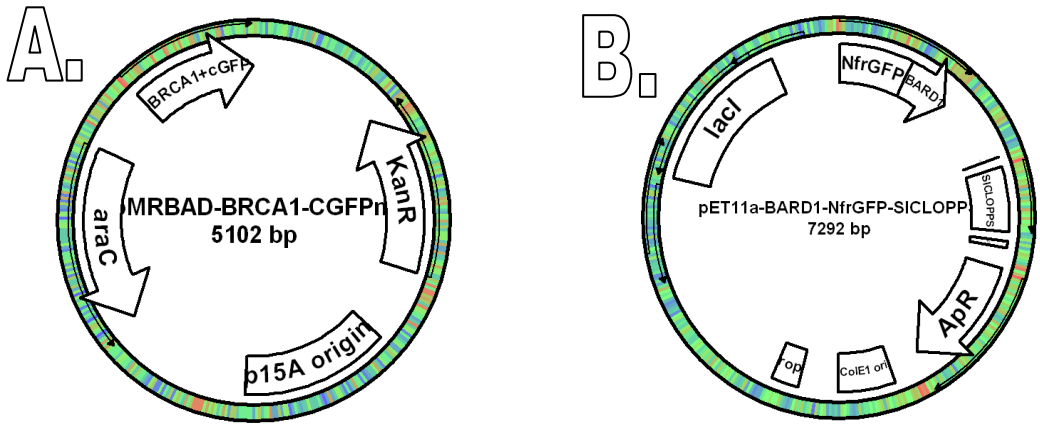

Figure 26. **A.** The BRCA1 half of the BRCA1/BARD1 plasmid screening system.
B. The BARD1 half of the BRCA1/BARD1 plasmid screening system.

The BARD1 plasmid did not contain many great restriction sites for library addition. The two sites chosen to place the library, AatII and EcoRI, only created an 80 base pair pop-put band. What this means this that no restrictions sites were placed within this pop-out, making a background digest impossible. The cloning was attempted once and the background was too high to ensure the transformation efficiency of the library could be adequately back calculated.

To fix this, a stuffer region from a different plasmid, pGFPuvbd1, was cloned between the EcoRI and AatII site. This resulted in many new choices for background digest sites.

Cloning a new stuffer fragment between the cloning sites eventually to be used for cloning in the SICLOPPS library into pET11-BARD1 was essential to ensure minimal background within the

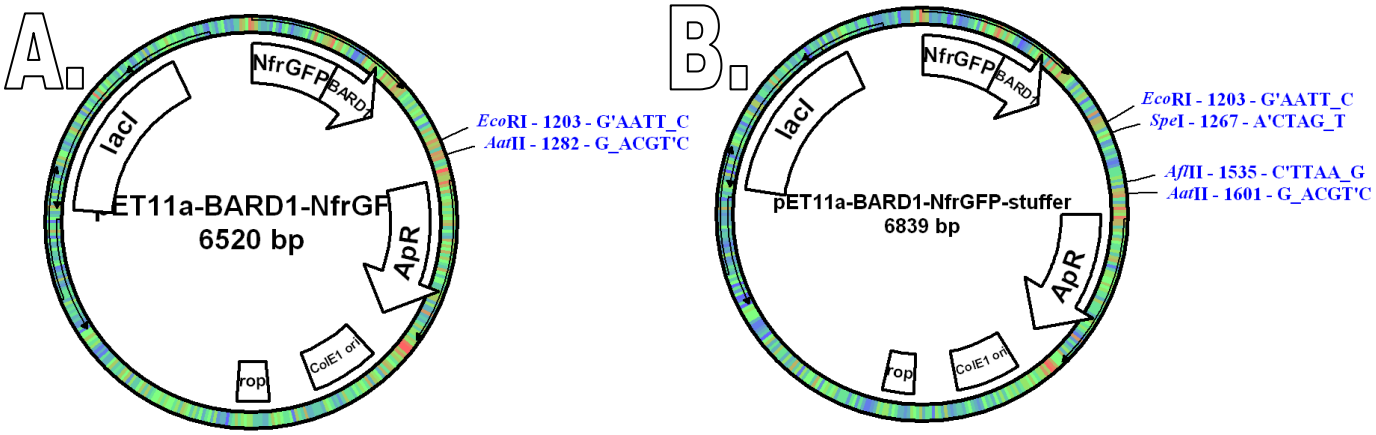

Figure 27. **A.** The parental vector for the BARD1-SICLOPPS cloning. Shown are the desired cloning sites for the library.
B. pET11a-BARD1-stfr with the new stuffer fragment, which is the new pop-out. SpeI/AflII are the background digest enzymes.

library. Cutting my stuffer out of the pGFPuvbd1 vector allowed for easy identification of which of the ligation products contained recyclized pGFPuvbd1 product. This was done using the UV Transilluminator, as cells containing that plasmid were fluorescent, and eliminating those cells. A clone was analytically digested and sequenced. It had the expected sequence and was then used as the cloning vector.

ii. Cloning of the SICLOPPS library into pET11a-BARD1-STFR

The SICLOPPS library, which was gifted from the Scott Lab in the pET28-SICLOPPS plasmid was grown and miniprepped. A PCR was done on the miniprep as described in the methods. The PCR was product was then digested using EcoRI and AatII restriction enzymes.

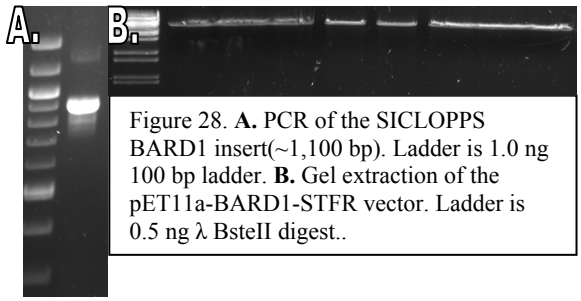

Figure 28. **A.** PCR of the SICLOPPS BARD1 insert(~1,100 bp). Ladder is 1.0 ng 100 bp ladder. **B.** Gel extraction of the pET11a-BARD1-STFR vector. Ladder is 0.5 ng λ BsteII digest..

At the same time, DH10B pET11A-BARD1-STFR

was grown, miniprepped, and digested twice with the same restriction sites. Both the vector and insert (figure 28) and ready to be concentrated, ligated, and screened. This is the next goal for this part of the project.

Chapter 4 – Conclusions and Future Directions

The usage of SICLOPPS molecules as modulators of protein function has great potential. Here I have described work attempting to fulfill the necessary background information for screening defective proteins for rescued activity upon introduction of chemical chaperones. Possible future directions include using FACS to sort out SICLOPPS molecules from the libraries that interfere with screening and then screening culled libraries in order to find drug leads. A similar proposition should occur with BRCA1, except that it might flow much more smoothly due to it being based on a positive phenotype screen.

Drug scaffolds discovered in this way could possibly lead to drugs that target specific mutations in proteins, a type of medicine where one drug can modulate every human malady. This style of drug research has been gaining more and more credence as time has passed by, with drugs currently on the market that target specific maladies common to specific groups of people, such as the targeting of African Americans with congestive heart failure with the drug BiDil.[94]

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